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(54) Title: ATP EXTRACTION REAGENT CONTAINING AMMONIUM VANADATE

(57) Abstract

An Ammonium Vanadate containing ATP extract reagent for extracting ATP from somatic tumor cells without disruption of the cell membrane from 2.6-4.6 millimolar of Ammonium Vanadate stabilizes the extracted ATP. Employment of the Ammonium Vanadate containing ATP extract reagent in the bioluminescent reaction method for assaying the effectiveness of chemotherapeutic agents against disassociated tumor cells constitutes an overall improvement in the method.

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ATP Extraction Reagent Containing Ammonium Vanadate

This invention relates to an ATP releasing reagent and in a preferred embodiment relates to an ATP releasing reagent adapted for employment in a bioluminescent assays of somatic tumor cells through measurements made of ATP extracted from the cells.

This invention also relates to an improved method for screening tumor cells to ascertain their sensitivity to chemotherapeutic agents.

BACKGROUND OF THE INVENTION

The selective determination of nucleotides in a sample through the firefly bioluminescent measurement of the adenosine triphosphate (ATP) content in a sample is a rapid and sensitive method for ascertaining the viability and number of cells in the sample. This bioluminescent measurement has been applied in the past to analytical test measurements that determine the number and viability of bacterial cells in a test sample, see U.S. Patent No. 3,745,090 and more recently to test measurements for the number and viability of either or both of somatic and microbial cells in a test sample, see U.S. Patent No. 4,303,752.

In vitro analytical tests on cellular ATP employing the firefly luminescent reaction with ATP have become sufficiently popular to engender commercial availability of ATP releasing reagents from laboratory supply houses as, e.g., FL-SAR™ (Sigma Chemical Co.), SOMALIGHT™ (Luminescence Analytical Laboratory) and PICOEX™ (Packard Instrument Co.). To conduct an assay the somatic cell sample is suspended in a solution containing an ATP releasing reagent composition, and

then the ATP content of the solution is measured through the firefly luminescence reaction.

In common, these ATP release reagents include a (non-toxic) wetting agent, e.g., non-ionic surface agents whose purpose is to render the cell membrane permeable to whatever relatively small molecules are present among the (cellular) constituents inside of the cell membrane. Thereafter, the small molecules diffuse out past the cell membrane barrier. In particular, ATP diffuses out past the cell membrane barrier so that the ATP content still inside of the cells and the ATP content in the aqueous medium surrounding the cells approaches an equilibrium that is in proportion to the number of (viable) cells suspended in the medium. Preferred non-ionic surface agents are the ethoxylated phenols and the fatty acid polygycol ethers. A more detailed discussion of the non-ionic surface active agents preferred for the ATP releasing reagent purposes with somatic cells and with microbial cells can be found in the U.S. Patent No. 4,303,752 to which reference is made. Preferred for the surface active agent are TRITON-X or NONIDENT P-40. It is important to note that the surface active agent is present in relatively low proportions, say 0.5 - 0.1%. In practice of this invention as in practice of 4,303,752 release of the ATP is accomplished without complete destruction of the cell membrane of the viable cells. Complete destruction of the cell membrane may release large molecular weight cellular constituents from inside of the cell including notable enzymes which would act on ATP.

In common, these ATP release reagent compositions known to the art also include a buffer composition whose purpose is to adjust the pH of the cell suspension in the (diluted) release reagent to some desired level. In the instance of ATP release reagents for somatic cells, any of the usual buffers,

e.g., tris may be employed but Hepes at pH 7.7 - pH 7.9 is a preferred buffer. Hepes which is N-[2-hydroxyethyl]piperazine N′ -[2-ethanesulfonic acid] does not interfere with the firefly bioluminescent reaction measurement of the ATP content in the buffered solution and, desirably, is adapted to buffer the suspension at a pH of about 7.8, which is an optimum level for the firefly bioluminescent reaction.

A variety of in vitro assay screening methods have been developed over the past two decades to test the applicability of chemotherapeutic drugs against tumors. The method for ascertaining the sensitivity of tumor cells to chemotherapeutic agents to which the practice of this invention pertains, comprises culturing a multiplicity of samples of tumor cells in the presence of different chemotherapeutic drugs at varying concentrations and then assaying the proportion of tumor cells that remained viable after 4-7 days in vitro using the firefly bioluminescent reaction for the assay. Ascertained thereby is the affect of different concentrations of the therapeutic drugs on the tumor cells. For the details of how this screening method has been conducted heretofore reference is made to Sevin et al., "Application of an ATP-Bioluminescence Assay in Human Tumor Chemosensitivity Testing", Gynecol. Oncol., 31:191-204 (1988). However, Sevin et al. disrupt the tumor cells. In the assay method of the present invention the cell membranes of the somatic tumor cells are not destroyed and, moreover, the firefly bioluminescent reaction is carried out on stabilized ATP (in solution).

BRIEF DESCRIPTION OF THE INVENTION

Briefly stated, the ATP release reagent of the present invention comprises a solution of known in the art components for ATP release reagents in art recognized proportions to render cell membranes

permeable without destruction of the cell membrane. Also present is NH_4VO_3 (Ammonium meta Vanadate) in proportions effective to inhibit any cellular ATPase enzyme(s) that might otherwise react away extracted ATP in the solution. In the absence of Ammonium Vanadate the level of ATP in the suspension of somatic tumor cells in the ATP release reagent composition rapidly decreases.

The range of concentrations of Ammonium Vanadate contemplated for the ATP release reagent composition of this invention is about 8-14 mM so that a proper concentration of the Ammonium meta Vanadate preferably in the range of about 2.6 to 4.6 mM (millimolar) appears in the cell suspension.

A separate aspect of this invention is an improved assay method for determining the sensitivity of tumor cells to chemotherapeutic agents, the improvement features comprising incorporating Ammonium Vanadate in ATP release reagent compositions that are adapted to release ATP from human tumor (somatic) cells without totally disrupting the cell membranes.

RATIONALE OF THE INVENTION

According to the method for assaying the sensitivity of tumor cells to chemotherapeutic agents described by Sevin et al. supra, extracting ATP from the tumor cells is done by a cell lysis technique using a strong acid such as trichloroacetic acid (TCA). However, lysis of the tumor cells releases ATPase enzymes from the cell into the ATP extract solution. Also this procedure requires another buffer reagent to achieve pH 7.8. Extracting ATP from viable cells according to practice of U.S. Patent No. 4,303,752 with ATP release reagents which do not totally disrupt the (tumor) cell membrane is believed to be a more advantageous method of securing an ATP content in solution that is in proportion to the viable cell count

of the sample, and such is done in the method of this invention.

In the instance of human tumor cells, it has been found, however, that the ATP extracted according to the technique of U.S. Patent No. 4,303,752 becomes eliminated rapidly from the ATP release reagent solution. Indeed ATP is not readily detectable in the somatic cell suspension release reagent solution within only about 15 minutes after extraction of the ATP into the ATP release reagent composition. It is believed that cellular ATPase enzyme(s) degrade the ATP. However, when effective amounts of Ammonium Vanadate have been included in the ATP release reagent, the ATP extracted into the cell suspension remains relatively stable. Measurements of extracted ATP can be obtained reproducibly for up to about four hours after the ATP extraction step.

Ammonium Vanadate seems to have a molecular structure similar to ATP, and apparently the Ammonium Vanadate binds with ATPase enzymes at an active site thereon. Whatever the reaction mechanism may be, Ammonium Vanadate inhibits the catalytic activity of ATPase enzymes on ATP, an inhibition which is known in the art, see Cantley et al., J. Bio. Chem., 252:7421 (1977).

The great advantage to stabilizing the ATP which has diffused from the tumor cells into solution in the ATP release reagent should be self-evident. The assay method of this invention is adapted to an essentially concurrent evaluation of a great many tumor cell samples so as to test varying amounts of different chemotherapeutic agents on the tumor. Preferably extraction is performed in all samples. Then the ATP in the samples are measured one by one. Stabilizing the ATP in each sample allows time for extraction and measuring the ATP from tumor cells from each sample, in proportion to the number of cells in the sample,

despite the widely differing tumor cells content in some of the multitude of samples. In addition, stabilizing the ATP content allows the time needed to manipulate the multitude of samples however necessary up to introduction of the luciferase-luciferin reagents into each sample, one by one for conduct of the firefly luminescence reaction assay. For carrying out the comprehensive assay method of this invention on human tumor cells, the ATP in solution in the particular tumor samples measured by the firefly bioluminescence reaction should be sufficiently stable to allow up to about 2.5 hours of delay between the addition of the NH_4VO_3 extraction reagent into the cell suspension medium and conduct of the firefly bioluminescent reaction.

DETAILED DISCUSSION OF THE INVENTION

A principal aspect of the invention is the presence of effective amounts of NH_4VO_3 in the ATP releast reagent. The Ammonium Vanadate content is in the range of about 2-6-4-6 millimolar and preferably in the range of about 3.0-4.0 millimolar for achieving an optimum stability of ATP counts. At concentrations about about 4.0 millimolar, a discernible decrease in counts has been observed. This is believed to be due to inhibition of the luciferase enzyme caused by excess NH4VO3 in the extracted sample. As expected, counts decrease as a function of decreasing NH_4VO_3 concentration and such occurs below the 3.6 millimolar concentration in the tumor cell suspension. The release reagent of this invention is particularly adapted for the release of ATP from human tumor cells without total disruption of the tumor cell membranes.

As already has been indicated herein, a second aspect of the present invention resides in an improved method for evaluating the sensitivity of somatic tumor cells to chemotherapeutic agents. The

early steps in the process generally follow the procedures described by Sevin et al., supra, but the final steps in this process involved extracting ATP from the cultured somatic tumor cells without totally disrupting the cell membrane into an NH_4VO_3 containing (ATP release reagent) solution, after which the solution is assayed for its ATP content by the firefly bioluminescence reaction.

Overall, the use of the ATP bioluminescence reaction to measure the effect of chemotherapeutic drugs against tumor cells cultured in vitro has been so simplified by practice of this invention, that tumor chemosensitivity assays can be performed rapidly and easily in a standardized format. In addition, the improved method of this invention provides an objective measurement determinative of sensitivity of the tumor cells to various drugs, using relatively few tumor cells in each sample.

BRIEF DESCRIPTION OF THE DRAWINGS

The assay method of this invention will now be described in relation to the attached drawings wherein:

Figure 1 is a flow sheet representation of the method;

Figure 2 is a graph showing luminometer counts 15 minutes after extraction; and

Figure 3 is a graph showing luminometer counts 30 minutes after extraction.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

Referring now to Figure 1 of the drawing, it may be seen that the first step of the method transforms a solid tumor specimen into a suspension of single cells or multi-cell aggregates of less than about 30 cells per aggregate. In the illustrated preferred mode of this invention, this transformation

is performed as described by Sevin et al., supra. First, the solid tumor specimen is minced into 1-5 mm pieces. Then, the minced tumor pieces are disassociated enzymatically into single cells and small size multi-cell aggregates by dispersing about 1 gram of minced tumor in sterile culture medium to which had been added about 1500 units per ml of DNase 1, about 2 mg/ml of collaganase and about 1 mg/ml of Dispase enzymes. Use of this particular combination of enzymes is a known in the art technique for disassociating solid tumor cell pieces into individual cells and (tiny) cell aggregates containing less than about 30 cells per aggregate.

The second step of the method comprises culturing a multiplicity of samples of the disassociated tumor cells in a standard nutrient medium for from 4-7 days. The growth medium and cultivation conditions is suitably described by Sevin et al. (McCoy's enriched media with 15% fetal calf serum at 37°C at 95% humidity for 6-7 days) in the presence, sample to sample, of varying concentrations of different chemotherapeutic agents. Of course, an appropriate number of control samples of the tumor cells are also cultured. One special consideration involved in culturing the disassociated tumor cells in the presence of chemotherapeutic drugs is that frequently normal cells will be present along with the tumor cells in some or all of the samples. Normal cells are an interfering substance in the assay method. ATP measurements derived from a normal cell's content in the samples are not desired. To the extent reasonably possible, normal cells should be eliminated from the test samples of tumor cells.

Fortuitously, normal cells are generally anchorage dependent cells. Therefore, culturing mixtures of disassociated normal and tumor cells inside of sample wells provided with wall surfaces to which

normal cells cannot anchor, will prevent proliferation of whatever normal cells are in the samples while allowing proliferation of the (non-anchorage dependent) tumor cells. Hopefully, all normal cells will die over the course of culturing period, while a significant number of tumor cells remain viable at expiration of the culture period. A preferred embodiment culture period is 4-7 days.

Agarose coated microtiter plates prepared as described in copending application, S.N. 07/651,940, filed February 7, 1991, is preferred for culturing the tumor cell samples. The agarose coated well bottom will prevent proliferation of normal cells therein. Of course, other techniques for placing an agarose coating on the well bottom of the commercially available polystyrene microtiter plate than through practice according to S.N. 07/651,940 may be used. Alternatively, polypropylene microtiter plates may be employed for culturing the tumor cell samples according to the method of this invention.

The third step in the bioluminescent method of this invention is extraction of the tumor cell cultures (without total disruption of the cell membrane) so as to release ATP from the tumor cells in proportion to the number of viable tumor cells in the culture. The resulting ATP solution is stabilized by the presence of Ammonium Vanadate in the solution. Other than the presence of Ammonium Vanadate therein, the ATP releasing reagent is comprised of known in the art ingredients. For example, one of the commercially available ATP release reagents FL-SAR™, SOMALIGHT™ or PICOEX™ may have a concentrated solution of Ammonium Vanadate added thereto generate 2.6-4.6 millimolar NH_4VO_3 in the cell suspension medium and then the solution is used to release ATP from the somatic tumor cell suspension following the details of the procedure set out in U.S. Patent No. 4,303,752. As a point of

preference, the ATP release reagent has been buffered with 0.1 - 0.35 M Hepes which is a relatively high buffer content. Typically, the pH of the tumor cells sample drops during cultivation to about pH 6.5, and the final pH (for the bioluminescent reaction) is pH 7.7 - 7.9. Preferably, the Hepes concentration in the cell suspension is about 0.125 M.

In the test study which is illustrated in Figures 2 and 3, the Ammonium Vanadate dissolved in distilled water was mixed with a 0.35 M Hepes solution and either NP-40 or Triton X-100 to 0.03-0.50% thereof by wt was incorporated to form the ATP releasing reagent. Typically, two parts of tumor cell suspension is mixed with one part of the ATP releasing reagent.

About 0.15% by wt of Triton X-100 or NP-40 seems to be a reasonable optimum and such constitute preferred embodiment wetting agents and the content thereof.

For conducting mass screening of therapeutic agents for effectiveness against tumors by the method of this invention it is important that the ATP extracted into the ATP release reagent should be relatively stable and not be degraded by ATPase enzymes indigenous to the tumor cells. Stabilizing the ATP dissolved in the medium in which the cells are suspended allows time to assay a great number of samples routinely. Desirably, the ATP content in the cell suspension medium should be stable enough to allow for quantitative reproductability of the ATP measurements made with the firefly bioluminescence reaction throughout about a 2 1/2 hour period following extraction of the ATP from each of the cultured tumor cell samples. Thus, stabilization of the ATP in solution in the cell suspension medium extract should solution through inclusion of Ammonium Vanadate in the ATP release reagent is important to practice of the overall test assay method of this invention.

For further understanding of this invention the following specific examples are provided.

Example 1

ATP extraction reagents containing 0.32 M Hepes buffer pH 7.8, varying amounts of Triton X-100 or NP-40 in the range of from 0.5 to 0.03% by wt with and without the inclusion of 10.86 mM NH_4VO_3 in the reagent were prepared and then used to extract ATP from ME180 ovarian tumor cells. The tumor cell extracts were counted by conducting the firefly bioluminescence reaction on Sample 15, 30 and 60 minutes after the ME180 tumor cells suspended in a fetal calf serum culture medium were first mixed with the ATP extraction reagent. In the absence of Ammonium Vanadate the ATP extracted from the tumor cells became rapidly eliminated from the reagent and little ATP was detected after only about 15 minutes, whereas good stability of the ATP counts for more than 60 minutes was evidenced when Ammonium Vanadate was present.

The results of the 15 and 30 minute study are provided in Figures 2 and 3. The rapid disappearance of ATP from unstabilized solutions is evidenced. The data shows that best ATP extraction is obtained with 0.13% by wt or more of the wetting agent.

Similar results have been obtained with other tumor cell lines, e.g., SW-948 colon and with tumors secured from patients. In all of the studies, the extracted ATP was stabilized by the presence of Ammonium Vanadate in the ATP extraction reagent.

It is believed that the optimum cell concentrations for being assayed with the preferred tumor cell extraction reagent is 10,000-80,000 cells/well of a 96 well flat bottom microwell plate in approximately 200 μl of culture media per sample.

Example 2

To carry out the comprehensive method of this invention a solid tumor specimen is minced and enzymatically disassociated as described in Sevin et al., supra, to which reference is made for details of the work up procedure. Briefly, the work up procedure is to disassociate the solid tumor as is illustrated in Figure 1 herein by mincing the same while the tumor cells are being bathed in a nutrient medium containing fetal calf serum, sufficient antibiotic to control possible microbial contamination and a tumor disassociation enzyme as already has been described herein. Then the minced tumor pieces are enzymatically cultured in more of the same nutrient solution mixture at 37°C until the tumor cells have visually been disassociated or as convenient, say for 8-16 hours.

Optionally, but preferably, ficoll-hypaque density gradient separation is used to reduce dead cell and erythrocyte contamination.

The disassociated tumor cells are washed, then resuspended in the fetal calf serum nutrient medium at a cell concentration of about $4x10^5$ cells per ml. About 0.1 ml of this suspension is added to each well of a 96 well agarose coated polystyrene culture plate. Parenthetically, it is noted that the best range of tumor cell concentration per well is from $2x10^4$ - $8x10^4$.

An essay for later in vivo treatment of the tumor is conducted by testing each proposed chemotherapeutic drug at the four concentrations corresponding to 12.5%, 25.0%, 50.0% and 100% of the estimated Peak Plasma Concentration (PPC) for the drug using triplicate cultures for each drug concentration. Fourteen chemotherapeutic drugs can be tested on two 96 microwell culture plates. The wells of the culture plates which remain open are used for MO "No Drug" and MI "Maximum Inhibitor" controls. Alternatively, each

proposed chemotherapeutic drug may be tested at eight concentrations, the additional concentrations being 1.56%, 3.13%, 6.25% and 200%. In this event, only ten chemotherapeutic drugs can be tested on two 96 microwell culture plates.

The drugs in appropriate concentration dissolved in the fetal calf serum nutrient medium are applied from a multi-channel pipette to the wells in the culture plates and then the microwell culture plates containing 0.2 ml per well of the drug dosed tumor cell culture samples are incubated for seven days in a humidified, 37°C, 5% CO_2 incubator. If rapid cell proliferation is evidenced, the incubation period should be decreased to 4-6 days.

The Ammonium Vanadate stabilized tumor cell extraction reagent formulated as described herein, is then added to each well in the culture plate at the rate of 0.1 ml per well and thoroughly mixed in to form a cell suspension in the mixture of culture medium and ATP extraction reagent that constitutes a Hepes buffered 0.05% by wt of Triton X-100, 3.62 mM NH_4VO_3 stabilized solution of ATP. The suspension is incubated about 20 minutes at room temperature before an aliquot thereof and the luciferin-luciferase counting reagents are mixed for measuring the ATP content. Parenthetically, it is noted that best results are obtained if the cell cultures are biolumimetrically counted within 60 minutes of the addition of tumor cell extraction reagent.

For actual counting of the ATP, .05 ml aliquots of the extracted tumor cell cultures are transferred into tubes and .05 ml per well of a known to the art luciferin-luciferase counting reagent for ATP measurement is injected into the aliquots one by one and the bioluminescent counts are measured over a 20 second period in a luminometer (a count integration time in the range of 15-30 seconds is recommended).

CALCULATION AND INTERPRETATION OF LUMINOMETRY RESULTS

The percentage of tumor cell growth inhibition for each test drug concentration is calculated by the equation:

$$1.0 - \frac{(TEST) - (MI)}{(MO) - (MI)} \times 100 = \text{Percent Inhibition}$$

(TEST) = Mean counts for test drug cultures

(MI) = Mean counts for Maximum Inhibitor controls

(MO) = Mean counts for No Drug controls

STRONGLY SENSITIVE: Mean inhibition for 3.13% - 25% PPC drug concentrations is 70% or greater. Associated with a 60-70% probability of a favorable response in vivo.

PARTIALLY SENSITIVE: Mean inhibition for 3.13% - 25% PPC drug concentrations is 50% - 70%. Associated with a low probability of a favorable response in vivo.

RESISTANT or STIMULATORY: Mean inhibition for 6.25% - 25% PPC drug concentrations is less than 50%. Inhibition of less than 15% may reflect a stimulatory effect of the test drug.

An assay as described above was made on a patient's Sigmoid Colon primary tumor using disassociated tumor cells at 1, 2 and $4x10^5$ cells ml/for the samples. The cell samples were continuously exposed to test drugs in vitro for seven days. Drugs were tested at 200, 100, 50, 25 12.5, 6.25, 3.13 and 1.56 percent of their estimated Peak Plasma Concentration (PPC) in vivo. Tumor growth inhibition was quantitated by ATP luminometry. Interpretations are based on mean percent inhibition at 25, 12.5, 6.25 and 3.13 percent of PPC. The results are tabulated below.

DRUG TESTED	PPC ug/ml	INTERPRETATION
5-FU/LEUCOVORIN	45.0/1.2	PARTIALLY SENSITIVE
5-FU	45.0	PARTIALLY SENSITIVE
LEUCOVORIN	1.2	RESISTANT
MITOMYCIN C	0.9	RESISTANT
ADRIAMYCIN	0.5	RESISTANT
VINBLASTIN	0.8	RESISTANT
CIS-PLATIN	2.5	RESISTANT
MELPHALAN	0.3	RESISTANT
METHOTREXATE	2.8	RESISTANT
BCNU	2.0	RESISTANT

CLAIMS:

1. An ATP release reagent which comprises an aqueous solution of a non-toxic surface active agent in proportions sufficient to render the cell membrane of viable cells permeable to relatively small molecules without disruption of the cell membrane, a buffer for adjusting the pH of a cell suspension to the pH value desired for a bioluminescent test assay of any ATP that becomes released from the cells into the ATP release reagent and Ammonium Vanadate dissolved in the ATP release reagent in amounts effective to stabilize any ATP that becomes dissolved in the ATP release reagent.

2. An ATP release reagent according to claim 1 wherein the non-toxic wetting agent comprises from 0.1 - 0.5% by wt of an ethoxylated phenol wetting agent.

3. An ATP release reagent according to claim 1 wherein the Ammonium Vanadate content therein is about 8 to 14 millimolar.

4. An ATP release reagent according to claim 1 buffered to about pH 7.8.

5. In the method for determining sensitivity of tumor cells to chemotherapeutic agents by:

culturing a predetermined number of disassociated tumor cells suspended in a growth medium in the presence of a chemotherapeutic agent;

extracting ATP from the cultured cells; and

thereafter measuring the quantity of ATP extracted with the firefly bioluminescence reaction, the improvement which comprises:

extracting ATP from the cultured tumor cells by contacting the cells with ATP release reagent comprised of an aqueous

solution of a non-toxic wetting agent in proportions sufficient to render the tumor cell membranes permeable to relatively small molecules without totally disrupting the cell membranes, a buffer for adjusting the pH of the tumor cell suspension to about pH 7.8 and Ammonium Vanadate in ATP stabilizing amounts.

6. A method according to Claim 5 wherein the Ammonium Vanadate content in the ATP extract solution is from 2.6 - 4.6 millimolar.

AMENDED CLAIMS

[received by the International Bureau on 6 October 1992 (06.10.92); original claims 1-6 replaced by amended claims 1-7 (2 pages)]

1. A reagent comprising:

about 0.03 to 0.50 percent by weight non-toxic surface active agent;

about 8 to 14 millimolar ammonium vanadate; and

buffer for adjusting pH to about 7.7 to 7.9, wherein said reagent extracts and stabilizes cellular ATP.

2. The reagent of claim 1 wherein said buffer contains Hepes.

3. The reagent of claim 2 wherein said buffer contains about 0.1 to 0.35 molar Hepes.

4. The reagent of claim 1 wherein said non-toxic surface active agent is Triton-X.

5. The reagent of claim 1 wherein said non-toxic surface active agent is Nonidet P-40.

6. A method for determining sensitivity of tumor cells to chemotherapeutic agents, comprising the steps of:

providing a predetermined number of disassociated tumor cells suspended in a growth medium;

culturing said cells in the presence of a chemotherapeutic agent, said cells being cultured under conditions sufficient for the cells to grow and divide;

extracting and stabilizing ATP from said cells by contacting the cells with a sufficient amount of reagent comprising non-toxic surface active agent, ammonium vanadate, and buffer for adjusting pH to about 7.7 to 7.9; and

measuring said ATP using a bioluminescent reaction.

7. The method of claim 6 wherein said reagent is contacted with the cells in an amount whereby the ammonium vanadate content is from about 2.6 to about 4.6 millimolar.

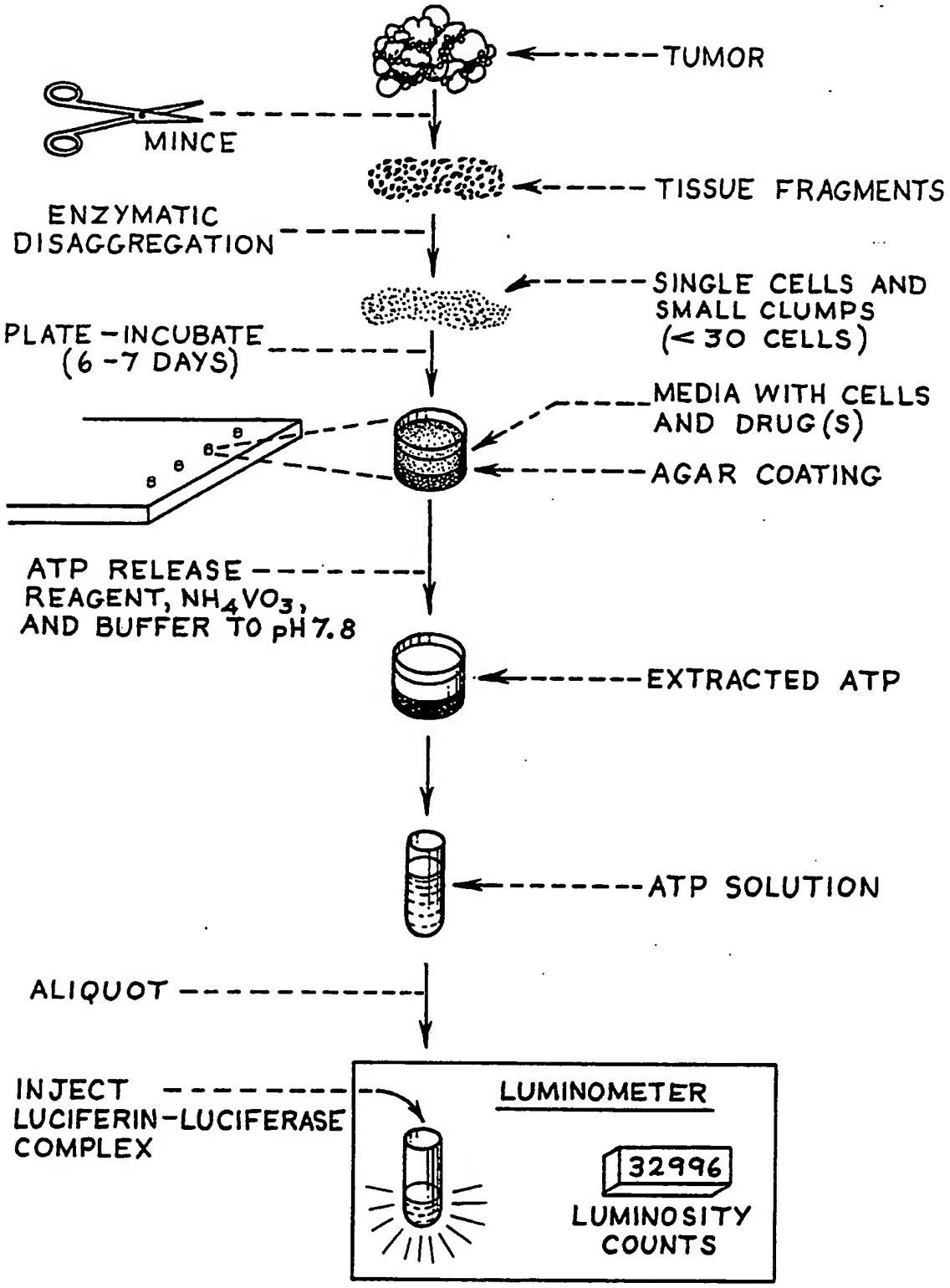

TUMOR
MINCE
TISSUE FRAGMENTS
ENZYMATIC DISAGGREGATION
SINGLE CELLS AND SMALL CLUMPS (< 30 CELLS)
PLATE - INCUBATE (6 - 7 DAYS)
MEDIA WITH CELLS AND DRUG(S)
AGAR COATING
ATP RELEASE REAGENT, NH_4VO_3, AND BUFFER TO pH 7.8
EXTRACTED ATP
ATP SOLUTION
ALIQUOT
INJECT LUCIFERIN-LUCIFERASE COMPLEX
LUMINOMETER
32996
LUMINOSITY COUNTS

Fig. 1

Fig. 2

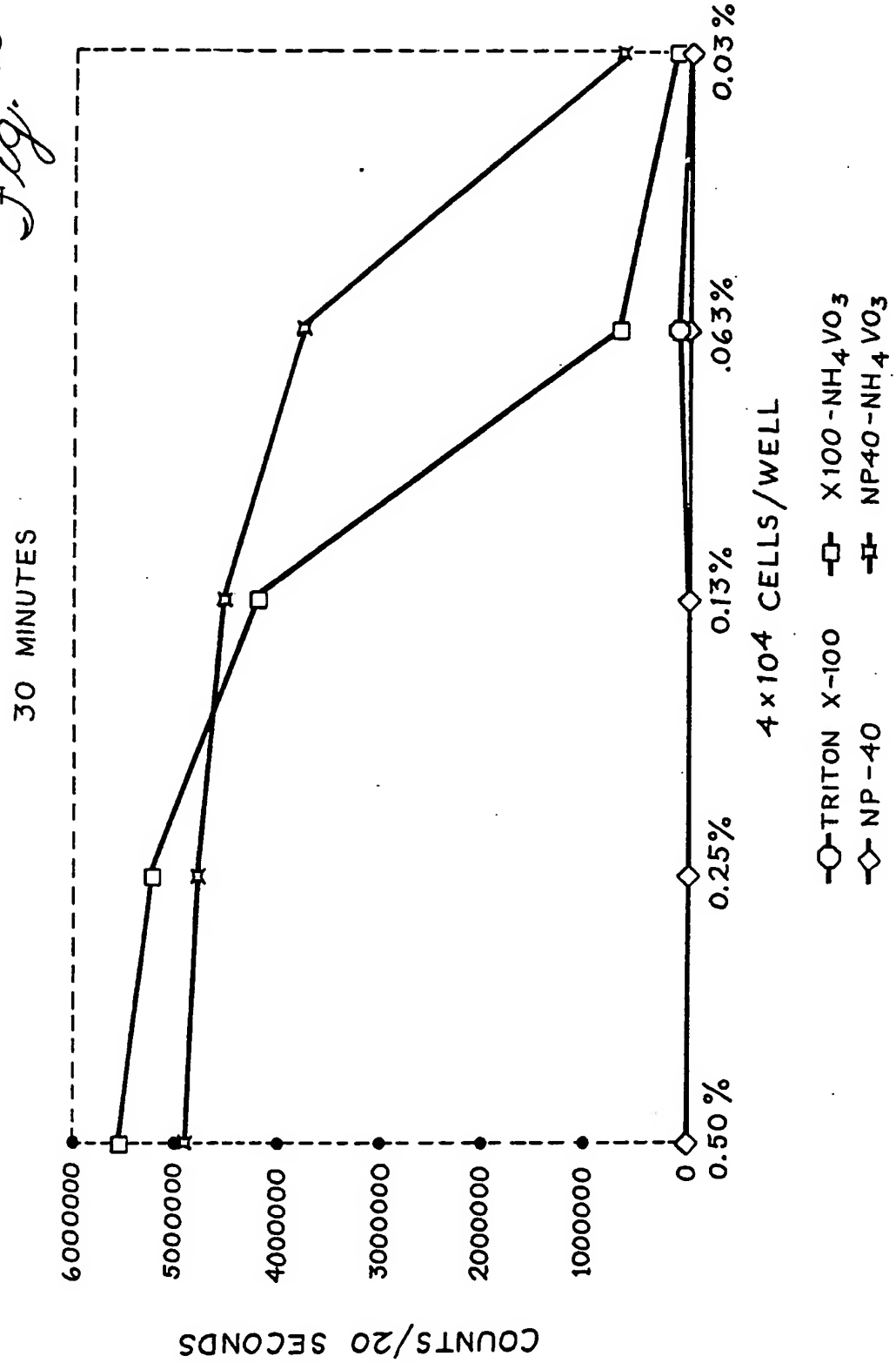
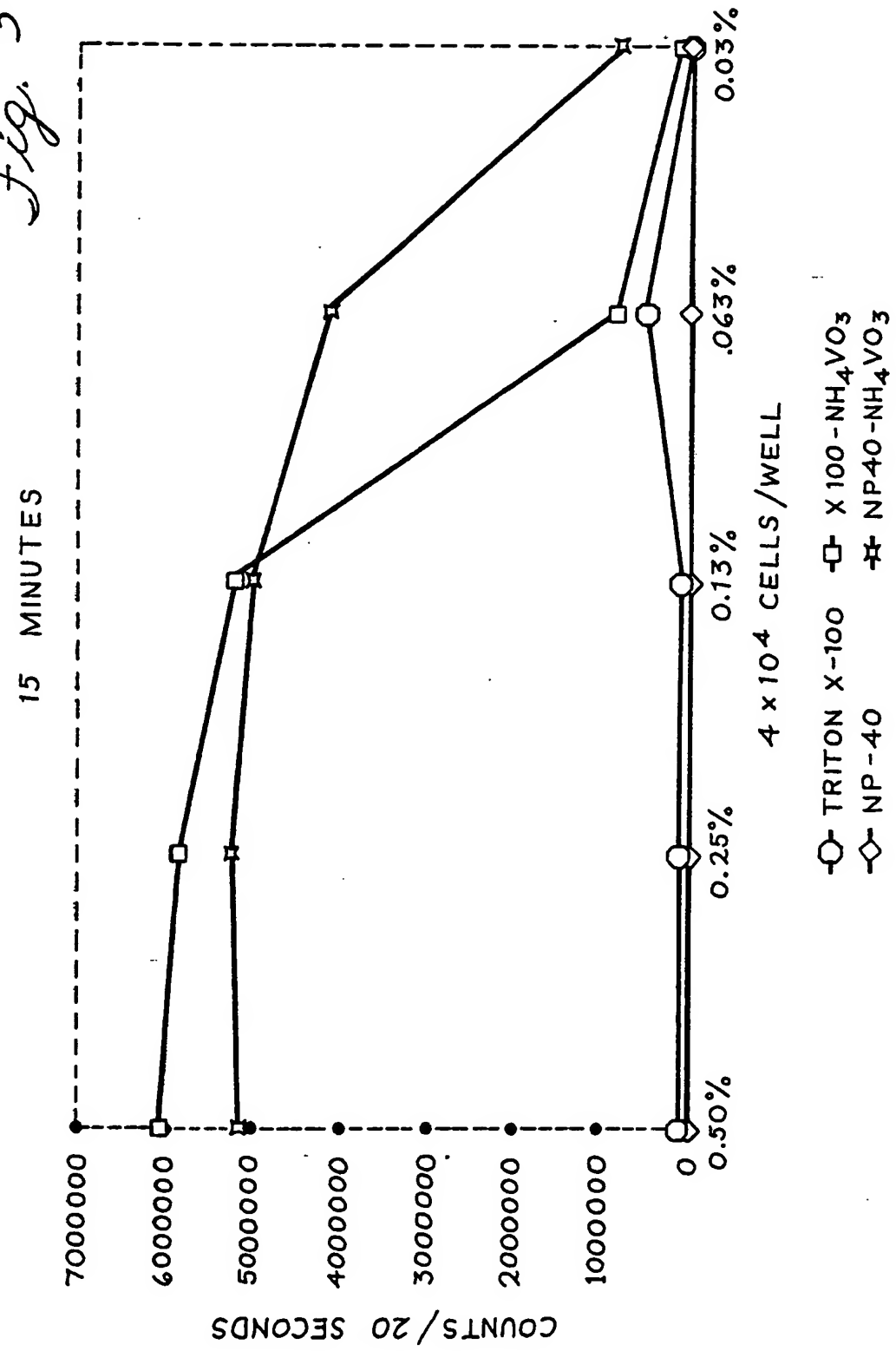

30 MINUTES
COUNTS/20 SECONDS
6000000
5000000
4000000
3000000
2000000
1000000
0
0.50 %
0.25%
0.13%
.063%
0.03%
4 x 10⁴ CELLS/WELL
TRITON X-100
NP-40
X100-NH4VO3
NP40-NH4VO3

Fig. 3


15 MINUTES
COUNTS/20 SECONDS
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
4 x 10⁴ CELLS/WELL
0.50%
0.25%
0.13%
.063%
0.03%
TRITON X-100
X100-NH4VO3
NP-40
NP40-NH4VO3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/04101

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 1/06; C12Q 1/66; C12Q 1/34
US CL : 435/8, 18; 536/27; 428/662

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/8, 18; 536/27; 428/662

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS
seach terms: ammonium (W) vanadate; Andreotti, Peter ?/in

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,303,752 (KOLEHMAINEN ET AL) 01 DECEMBER 1981, see claims 1 - 18.	1 - 6
Y	Journal of Biological Chemistry, Volume 252, No. 21, issued 10 November 1977, Cantley, Jr. et al., "Vanadate Is a Potent (Na,K)-ATPase Inhibitor Found in ATP Derived From Muscle", pages 7421 - 7423, see page 7422, column 2, paragraph 3.	1 - 6
Y	Gynecological Oncology, Volume 31, issued 1988, Sevin et al., "Application of an ATP-Bioluminescence Assay in Human Tissue Chemo-sensitivity Testing", pages 191 - 204, see entire document.	5 - 6

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
30 JUNE 1992	10 AUG 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	GARY L. KUNZ